

Adeno-associated Virus as a Gene Therapy Vector: Vector Development, Production and Clinical Applications

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Abstract Adeno-associated virus (AAV) has emerged as an attractive vector for gene therapy. AAV vectors have successfully been utilized to promote sustained gene expression in a variety of tissues such as muscle, eye, brain, liver, and lung [1–7]. As the significance of AAV as a gene therapy vector has been realized over the past years, recent developments in recombinant AAV (rAAV) production and purification have revolutionized the AAV field. It is now possible to produce high yields of vector (10^{12} – 10^{13} genome-containing particles per mL) that are free of contaminating cellular and helper virus proteins. Such vectors have been successfully used in preclinical applications in animal models such as those of hemophilia, lysosomal storage diseases and vision deficiency, all of which have shown therapeutic benefits from rAAV treatment. Clinical trials using rAAV2 for the treatment of hemophilia B, cystic fibrosis, alpha-1-antitrypsin deficiency, and Canavan disease have begun, and reports from these phase I trials support the safety seen in preclinical trials. Eventually, tissue-specific vectors that can potentially evade the immune system will be required to optimize success in gene therapy. In recent years, this has led to the development of retargeted rAAV2 vectors and the identification and characterization of new serotypes from human and nonhuman primates that could potentially achieve these goals. AAV virologists and gene therapists alike have just begun to scratch the surface in terms of the utility of this small virus in a clinical setting. In this chapter, we will

provide a comprehensive overview of the recent advances in rAAV vector production and purification, vector development, and clinical applications.

Keywords Adeno-associated virus · Adenovirus · Heparan sulfate proteoglycan · Transfection · Herpes simplex virus

1 AAV Biology

AAV is a member of the parvovirus family. Parvoviruses are among the smallest of the DNA animal viruses with a virion of approximately 25 nm in diameter composed entirely of protein and DNA. AAV has been classified as a dependovirus because it requires coinfection with helper viruses such as adenovirus (Ad) or herpes simplex virus (HSV) for productive infection in cell culture [8, 9].

The AAV2 genome is a linear, single-stranded DNA molecule containing 4679 bases [10]. The wild-type (wt) AAV genome is made up of two genes that encode four replication proteins and three capsid proteins respectively and is flanked on either side by 145 bp inverted terminal repeats [10, 11], as shown in Fig. 1. The larger replication proteins, Rep 78 and 68, are splice variants originating from the p5 promoter. Rep 68 and 78 are multifunctional and play a role in almost every aspect of the life cycle of AAV such as transcription, viral DNA replication and site-specific integration into human chromosome 19. The small replication proteins, Rep 40 and 52, have been characterized as important for DNA packaging into the preformed viral capsid within the nucleus [12, 13]. The virion is made up of the three capsid proteins Vp1, Vp2 and Vp3 in a ratio of 1 : 1 : 8 respectively. The capsid proteins are produced from the same open reading frame (ORF) but utilize different translational start sites. Vp3 is the most abundant subunit in the virion and is used for receptor recognition at the surface of cells defining the tropism of AAV. A phospholipase domain essential for viral infectivity has been identified in the unique N-terminus of Vp1 [14, 15]. The functional significance of Vp2 remains ambiguous, but recent studies have shown that it is possible to make viable virus lacking Vp2 in a recombinant background [16]. The biology of AAV2 has been extensively reviewed elsewhere [17, 18]. The current chapter is limited to issues concerning the use of AAV as a gene therapy vector.

One of the distinctive features of AAV replication has been the requirement for coinfection of the cell with a helper virus. In vitro, in the absence of helper virus, AAV can establish a latent infection by integrating into a unique site on human chromosome 19 [19–21]. Infection of cells latently infected with AAV by Ad or HSV will rescue the integrated genome and begin a productive infection [21–23]. While Ad early gene products serve as helper functions for AAV replication, late Ad gene products have been found to be unnecessary.

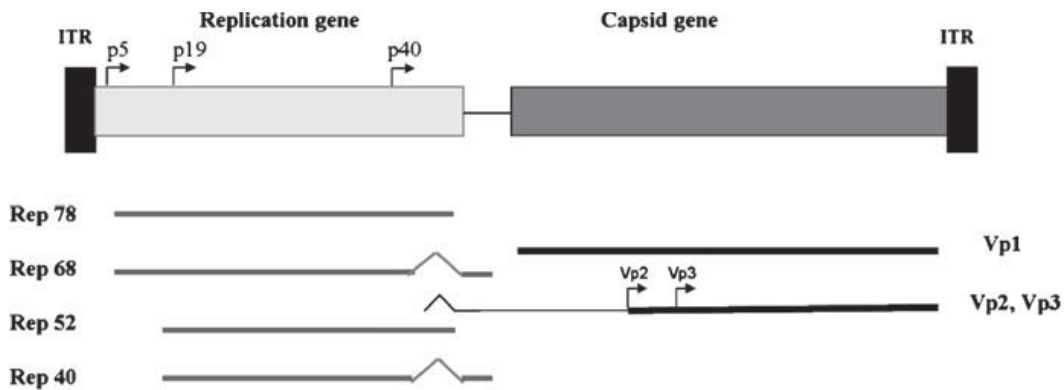


Fig. 1 Genomic structure of wild-type AAV. The p5 promoter initiates transcripts for Rep 78 (*when unspliced*) and Rep 68 (*spliced*). The p19 promoter initiates transcripts for Rep 52 (*unspliced*) and Rep 40 (*spliced*). Both Rep 52 and 40 products are in-frame with the Rep 78 and 68 products. The p40 promoter initiates all three capsid mRNAs. Vp1 is produced by an alternative splicing event. Using the same mRNA as Vp3, Vp2 is produced from an upstream alternative ACG start codon

The four Ad proteins required for helper function are the early region E1A, E1B, E4, and E2A. The synthesis of the Ad virus-associated (VA) RNAs is also required. The E1A protein is responsible for transactivation of AAV gene expression [24] and the E4 and E1B regulate the expression of AAV genes by aiding in mRNA transport to the cytoplasm [25] and aid in DNA replication by stopping the cell cycle in the G2 or S phase [25–28]. The E2A DNA binding protein aids in activating transcription from AAV promoters [29, 30] and may be involved in AAV DNA replication. By preventing the interferon-induced host cell shutoff mechanism of translation, Ad VA RNA assists the initiation of AAV protein synthesis.

Herpesviruses have also been identified as helper viruses for productive AAV replication. In order to determine the HSV genes essential for helper activity, Weindler et al. (1999) produced different HSV mutants defective in individual HSV replication genes [31]. Similar to Ad, AAV replication is not dependent upon the expression of late HSV genes. Genes encoding the helicase-primase complex (UL5, UL8, and UL52) and the DNA-binding protein (UL29) have been found to be sufficient to mediate the helper effect.

2

Vector production

AAV has developed into one of the most important and safest viral gene delivery vectors in the field of gene therapy. The simplicity of the AAV genome allows for the straightforward design of rAAV vectors to deliver transgenes of interest. The inverted terminal repeats flanking either side of the genome are the only cis-acting elements necessary for genome replication, integration

and packaging into the capsid. rAAV can therefore be produced by replacing the replication and capsid genes with a promoter and gene of interest (vector DNA). The rep and cap genes that encode the replication and structural proteins are expressed in trans from a different plasmid lacking ITRs. The separation of these genes from the vector DNA (containing the ITR packaging signal) is critical for circumventing the formation of wtAAV. As mentioned previously, helper functions from adenovirus or herpes simplex virus are necessary for rAAV production.

In the past, the inability to produce high-titer stocks of rAAV without contamination from helper virus and wtAAV impeded the use of AAV for clinical applications. The initial protocol for production of rAAV involved cotransfection of cells with vector DNA and a packaging plasmid expressing the AAV rep and cap genes followed by infection with Ad at a low multiplicity of infection (moi) [32]. Yields of between 10^2 and 10^3 viral particles/cell are characteristic of this method and the stocks are often contaminated with helper virus. Several improvements on this method have been made over the years that have resulted in increased vector yields. The first was the recognition that low levels (below a certain threshold) of capsid proteins can decrease rAAV production [33]. The second was that reduced levels of Rep 78 expression result in higher capsid protein synthesis, increasing rAAV titers [33–36]. The third was replacement of the standard adenovirus infection by introducing essential Ad helper genes on a plasmid for DNA transfection [37, 38]. The fourth and fifth were the introduction of stable packaging cell lines [39] and the baculovirus system [40] to produce rAAV that are amenable to scaling up production. The majority of these improvements resulted in rAAV preparations that were free of contaminating Ad and its structural proteins. The next few sections will describe the aforementioned improvements used to increase rAAV titers and scale-up production in detail.

2.1

Transient transfection methods

The standard transient transfection methods described here utilize the calcium phosphate precipitation protocol reported by Chen and Okayama in 1987 [41]. The first demonstration that purified Ad DNA could supply helper functions to rAAV production was provided by Ferrari et al. in 1996 [42] and this finding led to a number of plasmid-based methods to produce high titer rAAV.

Xiao et al. introduced the triple transfection method in 1998 [38]. They generated plasmids pXX2 and pXX6. The pXX2 helper plasmid contains the wtAAV rep and cap genes essential for AAV production. The pXX6 Ad helper plasmid contains the Ad genes necessary for rAAV production and lacks the genes necessary for Ad virion production and the ability to produce Ad structural proteins. The ATG start site of Rep 78 and 68 was mutated to ACG to

down-regulate their expression [35] and an additional p5 promoter element was cloned downstream of the Cap gene. It has been shown by a number of studies [43–46] that the lack of the p5 promoter cis-acting element causes down-regulation of the p19 and p40 promoters. They reasoned that an additional copy of the p5 promoter element may act as an enhancer for transcription

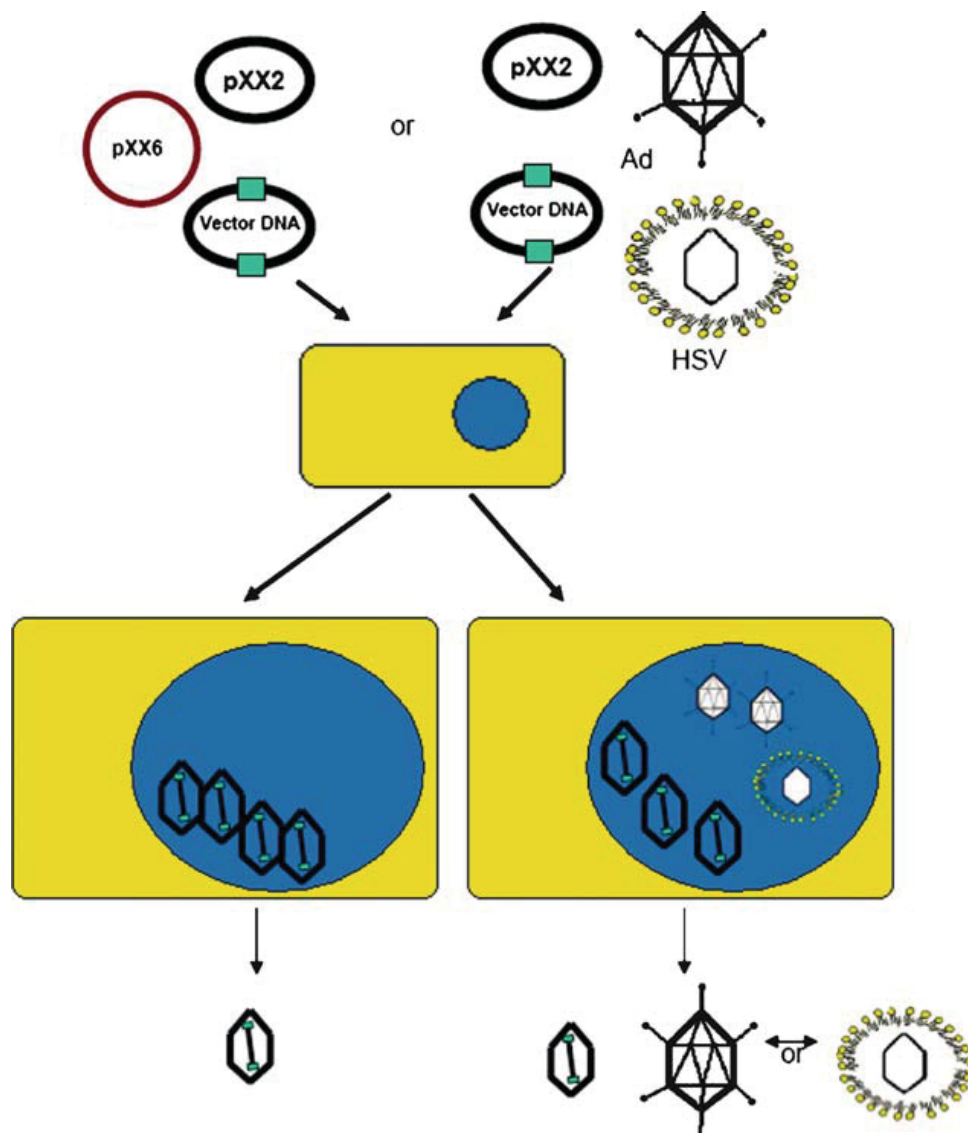


Fig. 2 A A schematic diagram of transfection methods used to produce rAAV with and without the use of helper virus. The triple transfection method developed by Xiao et al is depicted in the *left* of the transfection scheme. Three plasmids pXX6 containing the Ad helper genes, pXX2 containing the replication and capsid genes, and vector DNA (containing the ITRs flanking the transgene of interest) are transfected into cells. Cells are incubated 48–72 hours prior to isolating rAAV. The triple transfection method produces rAAV only while helper virus is found with rAAV when infected into cells for helper function

from the p19 and p40 promoters, which was illustrated in their studies. Utilizing the pXX2 plasmid and vector DNA followed by Ad infection, rAAV titers were increased 15-fold over its parental plasmid, pAAV/Ad [32]. Transfection of the pXX6, pXX2 and the vector plasmid (Fig. 2A) resulted in high-titer rAAV vectors with yields of approximately 1000 transducing units or 10^5 viral particles/cell without detectable contaminating Ad proteins and wtAAV.

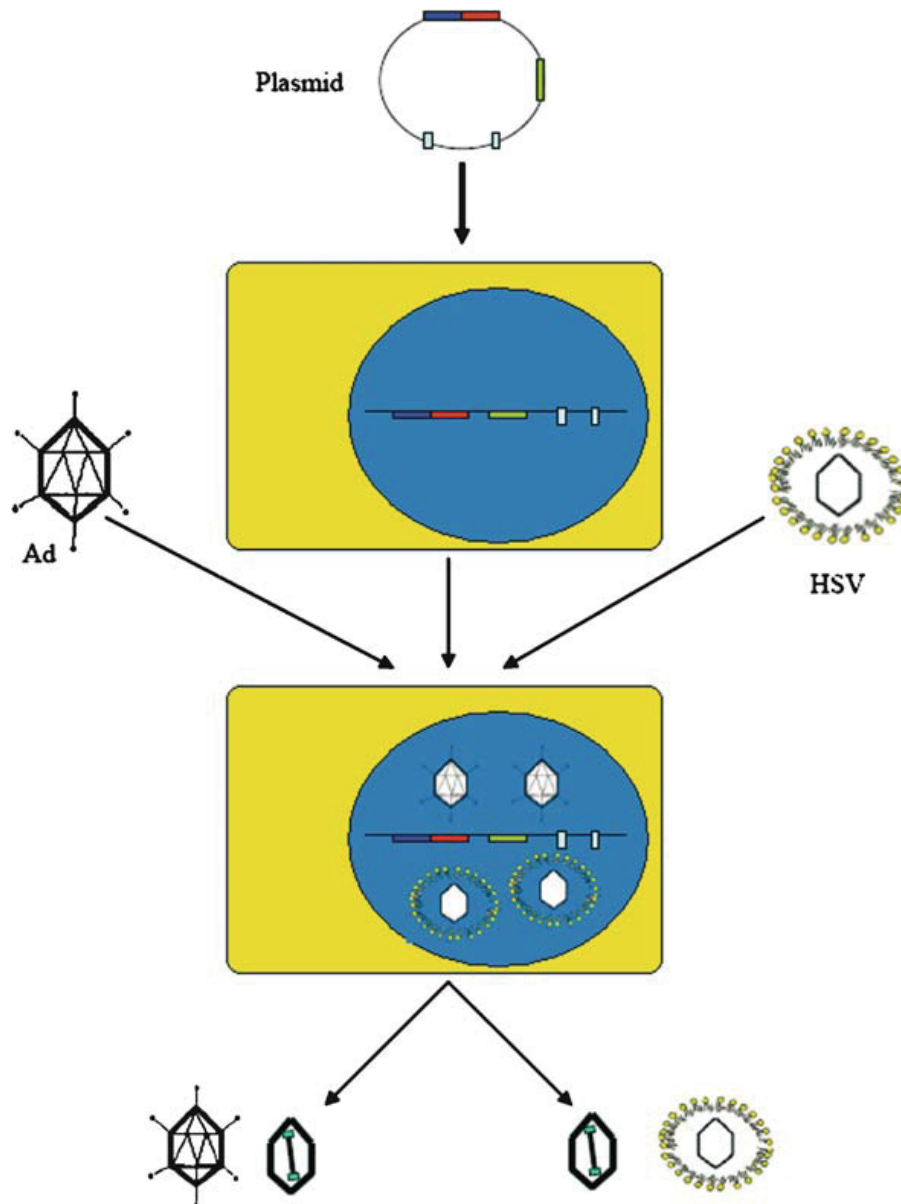


Fig. 2 B A schematic diagram of the production of rAAV through the use of a stable producer cell line. A plasmid containing rep (*blue box*), cap (*red box*), selectable marker (*green box*) and the transgene of interest flanked by ITRs (*light blue*) are transfected into cells. Cells that contain integrated copies of the plasmid are selected using a suitable media and subsequently infected with Ad or HSV to produce rAAV

In a similar study, a single plasmid containing the Ad helper and the rep and cap genes (pDG) was developed [36]. In this construct, the rep p5 promoter was replaced with the MMTV-LTR (mouse mammary tumor virus long terminal repeat) promoter to decrease the rep 78/68 expression levels and in turn increase the production of the capsid proteins. Cotransfection of 293 cells with pDG and AAV vector plasmid was sufficient to produce rAAV at titers of approximately 50–500 infectious particles/cell or 5×10^3 to 5×10^4 viral particles/cell. Allen et al. were able to limit the number of Ad helper genes to just one, the E4orf6 gene, to produce rAAV by utilizing helper AAV plasmids containing the MT (mouse metallothionein) and CMV (cytomegalovirus) promoters in replacement of the p5 and p40 promoters respectively. Yields of approximately 10 000 rAAV particles/cell were achieved after quadruple transfection of plasmids MT-rep, CMV-cap, vector DNA and the E4orf6 into 293 cells. Other studies utilizing variations of the transfection method with Ad mini-plasmids yielded titers of approximately 14 000 rAAV particles/cell [37, 47, 48].

Zhang et al. utilized DISC (disabled single-cycle virus) HSV (PS1) containing a deletion of the glycoprotein H (gH). This virus is infectious only if propagated in a complementing cell line [49], similar to Ad mutants grown on complementing 293 cells. They were able to use the DISC-HSV as a helper for rAAV replication, and have simulated to some extent the amplification of the rep and cap genomes seen in wtAAV infection by incorporating both these and vector sequences in HSV amplicons. In this system, increased production of AAV rep and cap proteins translated to an improved recovery of rAAV. When transfecting the HSV amplicon into BHK cells followed by infection with DISC-HSV helper virus, approximately 28 000 DNase resistant rAAV particles/cell are produced [50]. All of the above-described systems have allowed researchers to routinely generate AAV vectors for preclinical and phase I clinical trials.

2.2

Stable cell lines for production of AAV

Packaging cell lines are currently being developed for the large-scale production of rAAV. Vincent et al. were the first to demonstrate that a cell line stably expressing rep and cap could be used to produce rAAV. This method involved transfection of the rAAV genome and titers were low [120].

rAAV producer cell lines are generated by transiently transfecting the desired plasmid or plasmids containing the vector DNA with or without the essential rep and cap genes. A selectable marker is also present on each plasmid. After the cells have been transfected and selected in suitable growth media, stable cell lines are screened for viral production by Ad infection. The clones that produce the most rAAV are used as producer cell lines. Thus, this method can be used to produce rAAV without the need to transfect plasmid

DNA into cells. Infecting these cell lines with Ad or HSV is sufficient to generate rAAV (Fig. 2B). A HeLa cell line was generated as described above and infected with wtAd to produce rAAV [39, 50, 51]. This method was shown to produce approximately 40 000 particles/cell. Although this approach yields high titers of rAAV, a disadvantage of this approach is that new producer cell lines must be generated for each transgene construct with the risk of Ad contamination.

Many variations of this approach have also been tested. Gao et al. infected a HeLa cell line (B50) that has the rep-cap genes with endogenous promoters stably integrated. rAAV was produced in a two-step process [52]. The B50 cells were infected with an Ad defective in E2b to induce rep and cap expression and provide helper functions, followed by a hybrid virus in which the AAV vector is cloned in the E1 region of a replication-defective Ad. This method results in a 100-fold amplification and rescue of the AAV genome with a rAAV yield of 55 000 particles/cell that is wtAAV-free. The advantage of this method is that stable cell line selection is not necessary when producing rAAV with varying transgenes. In a similar study, but lacking a producer cell line, Zhang et al. generated rAAV using only Ad [53]. All components required for rAAV production were delivered to 293 cells by three Ad vectors. They estimated that 5.2×10^4 to 3×10^5 DNase resistant particles per cell are produced from this approach. A potential limitation of this approach is the possibility that some of the Ad may contaminate the rAAV preparations leading to the possibility of generating replicative rAAV in host cells.

The development of a stable cell line has not been limited to the use of Ad helper only. A similar production system for HSV-1 carrying the AAV rep and cap genes (d27.1-rc) has been described by Conway et al. [54]. This rHSV-1, although replication defective, expresses the HSV-1 early genes required for rAAV replication and packaging. 293 cells harboring an integrated copy of vector DNA are infected with the rHSV-1 to produce rAAV with the transgene of interest. rAAV vector yields were reported to be greater than 10^4 particles/cell.

Continued development of stable cell lines including integrated inducible systems to produce rAAV have been tested [55, 56]. Qiao et al. developed the “dual splicing switch” where an intron and three polyadenylation sequences are inserted into the protein coding region of the rep gene and flanked on either end by LoxP sites. The intron and polyadenylation sequences disrupt all four Rep genes because they contain transcription termination sequences. As a result, all of the Rep transcripts are prematurely terminated and the genes inactivated making this system viable in 293 cells expressing the E1 Ad gene. Removal of the terminator sequences by Cre protein reactivates the transcription of all four Rep proteins. The aforementioned cassette (derived from pXX2) along with the vector DNA cassette are stably integrated in 293 cells. Since 293 cells already stably express E1A and E1B genes, an adenovirus with E1A and E1B deleted would provide sufficient helper functions

for rAAV production. Large-scale vector productions (20×5 -cm plates) with GFP and minidystrophin cell lines yielded 6.8×10^{13} and 1.24×10^{14} vector genome particles per mL respectively. These yields are 30–60 fold higher in titer over the previously described triple transfection method with pXX2 and pXX6 [38].

2.3

The baculovirus system

The use of the baculovirus system to produce high titers of rAAV2 has been demonstrated [40, 57]. The recombinant baculovirus derived from the *Autographa californica* nuclear polyhedrosis virus, widely used for large-scale production of heterologous proteins in cultured insect cells, was used in this study. rAAV were produced by coinfecting three recombinant baculoviruses (a Rep-baculovirus, a VP-baculovirus, and an AAV ITR vector baculovirus) into suspension Sf9 cells. The yields from this system were compared to those obtained by transfection of 293 cells [36]. 4×10^8 cells were used for rAAV production by infecting 200 ml of Sf9 cells or transfecting twenty 15-cm plates of 293 cells. PCR results from this study illustrated that 1.8×10^{13} viral genomes (vg) were produced in Sf9 cells compared with 2×10^{12} vg by transfection of 293 cells corresponding to approximately 5000 vg/293 cell and 50 000 vg/Sf9 cell. Along with the baculovirus system, the HSV- and Ad-infected packaging cell lines represent the most promising for GMP scale production.

In summary, numerous successful methods producing high titers of rAAV have been developed. Each method has its advantages and disadvantages depending on its application. More importantly, as better methods of producing rAAV continue to be developed, the generation of purification methods that can be used to obtain high concentrations of uncontaminated vector becomes essential. The next section details recent advancements in vector purification.

2.4

Vector purification

The method used for the purification of rAAV particles is another critical step for the assessment of vectors in preclinical and clinical trials. It is essential to have purification methods that are suitable for purifying high titers of AAV from cell homogenate free of contaminating cellular proteins and/or helper virus. The traditional method used to purify AAV from infected or transfected cells has been density equilibrium gradients (cesium chloride, CsCl). Because cesium atoms are heavy, concentrated solutions of CsCl can form density gradients after only a few hours of ultracentrifugation. The buoyant density of a molecule is defined as the concentration of CsCl, in g/cm^3 , at that exact point in the density gradient at which the molecule floats [58].

The density of AAV packaging a wild-type size genome is approximately 1.41 g/cm^3 . This method allows for the physical separation of full particles (AAV packaging a genome) from empty particles based on their differences in density. Contents in the CsCl gradient are traditionally collected from the bottom of the gradient by dripping. Methods such as dot blot analysis or quantitative PCR, used to assess the presence of the viral genomes, are then carried out on each fraction of the gradient to identify the fraction(s) that contain AAV. There are many disadvantages to using CsCl gradients to purify AAV. Two or three rounds of CsCl centrifugation must be carried out to get purified AAV. Dialysis of CsCl fractions containing AAV against a physiological buffer is necessary prior to *in vivo* analysis because CsCl can exert toxic effects on animals in the study. This process can take up to two weeks from start to finish. It has also been shown that, even after CsCl purification, 1% of the input infectious Ad and/or Ad proteins can still be found as contaminants. Based on these limitations, a protocol that could reduce the preparation time without sacrificing purity and quality of AAV preparations was needed.

Research efforts in this area resulted in an immunoaffinity column to purify AAV2 particles [36]. The monoclonal antibody A20 used in the affinity columns, which recognizes assembled AAV2 capsids but not unassembled capsid proteins, has been characterized previously [59]. In this study, a known amount of infectious AAV2 in the cell homogenate was passed over the affinity column resulting in 70% recovery of the starting virus. The A20 affinity column was also used to purify a wtAAV2 stock that was generated using Ad. Dot blot hybridization of the starting material and the eluted virus from the column using a probe specific for the Ad genome showed that contaminating Ad could be completely removed from the AAV2 stock. The purity of the eluted AAV2 particles was determined to be 80% by utilizing polyacrylamide gels followed by silver staining. Affinity column purification allows for fast and easy preparation of concentrated and purified AAV2 stocks in a single step.

Zolotukhin et al. developed a novel way of purifying rAAV from crude lysates that involved the use of iodixanol gradients and heparin column purification [60]. The density of macromolecules in iodixanol is different from that in CsCl. Purified rAAV was found to have a density of 1.266 g/cm^3 in a continuous iodixanol gradient, which is equivalent to a 50% solution of iodixanol. To purify rAAV utilizing iodixanol, step gradients of 15%, 25%, 40%, and 60% were generated and the crude lysate was placed on top of the 15% step. After an hour of centrifugation, the majority of the rAAV bands were within the 40% density step. Approximately 75–80% of the rAAV in the crude lysate is recovered in the iodixanol fraction. For further purification after isolation of rAAV from iodixanol gradients, heparinized columns were used to determine if rAAV is capable of binding these columns. Heparan sulfate proteoglycan (HSPG) was recently identified to be a cell surface receptor for AAV2 [61]. HPLC chromatography utilizing UNO-S1 heparin columns was capable of yielding a rAAV product that is greater than 99% pure based on polyacrylamide gel electrophoresis and silver

staining. Utilizing the calcium phosphate transfection protocol with pDG and vector plasmids, yields of 2×10^{10} infectious particles per ten 15 cm plates or approximately 3.1×10^8 293 cells can be acquired. When coinfecting with Ad to produce rAAV, the authors found the presence of less than 1% (0.0017%) of the starting Ad contaminating the heparin purified rAAV. A five-fold higher recovery of rAAV particles and a greater than 100-fold increase in infectivity was obtained using this method of purification when compared to the traditional CsCl gradient purification. The main disadvantage to using this system of purification is that AAV must have the ability to bind HSPG (currently only AAV2 and AAV3).

At this point, the only purification method that can be utilized to purify all of the new and novel AAV serotypes is the CsCl gradient method. Recently, methods have been developed that have the ability to purify all AAV serotypes by utilizing ion exchange chromatography [62, 63]. Gao et al. utilized cation and anion chromatography to purify rAAV in their B50/hybrid cell line production method. They utilized POROS 20 HE as the first column (cation) and the POROS 50 PI (anion) as the second column. The POROS 20 HE column was used to bind AAV from the cell lysate. This column alone was able to remove a majority of the infectious Ad used to produce rAAV, but not to the levels needed for clinical use. Therefore, the incorporation of a second column (anion exchange resin) was used for the purpose of binding the remaining Ad and cellular contaminants while allowing rAAV to flow through. Particle to infectious particle ratios were compared to other methods of purification and these ratios were between 4 and 40 for the two-column method. Based on these numbers, this was much lower than the B50/hybrid-produced rAAV purified by CsCl (150–200) and the 293 triple transfection method purified by CsCl (400–1000) in their hands. Kaludov et al. developed a similar method in order to purify rAAV serotypes 2, 4 and 5 using an HPLC anion exchange method. Conditions such as pH of buffers and concentration of the detergent octylglucopyranoside (OGP) were critical when purifying each serotype. The authors found that the particles were purified more than 60 000-fold from the crude lysate using this two-step procedure. Freeze-thaw lysate was solubilized with OGP and passed over the PI anion exchange resin column, enriching the virus over 200-fold. The PI eluate still contained a number of contaminating proteins. The eluate was then further purified and concentrated using a high molecular weight retention filter. The end-product consisted of over 90% AAV particles. Particle to infectious particle ratios were reported to be similar to those achieved by affinity column chromatography. Most importantly, this method was found to be amenable to scaling up. Using a 90 mL PI column, 10^{13} rAAV particles can be purified in three hours.

In summary, numerous methods have been developed for the production and purification (Fig. 3) of AAV that are focused on acquiring high vector yields free from contaminating cellular and helper virus proteins and are amenable to scaling up for human clinical trials. Needless to say, as better

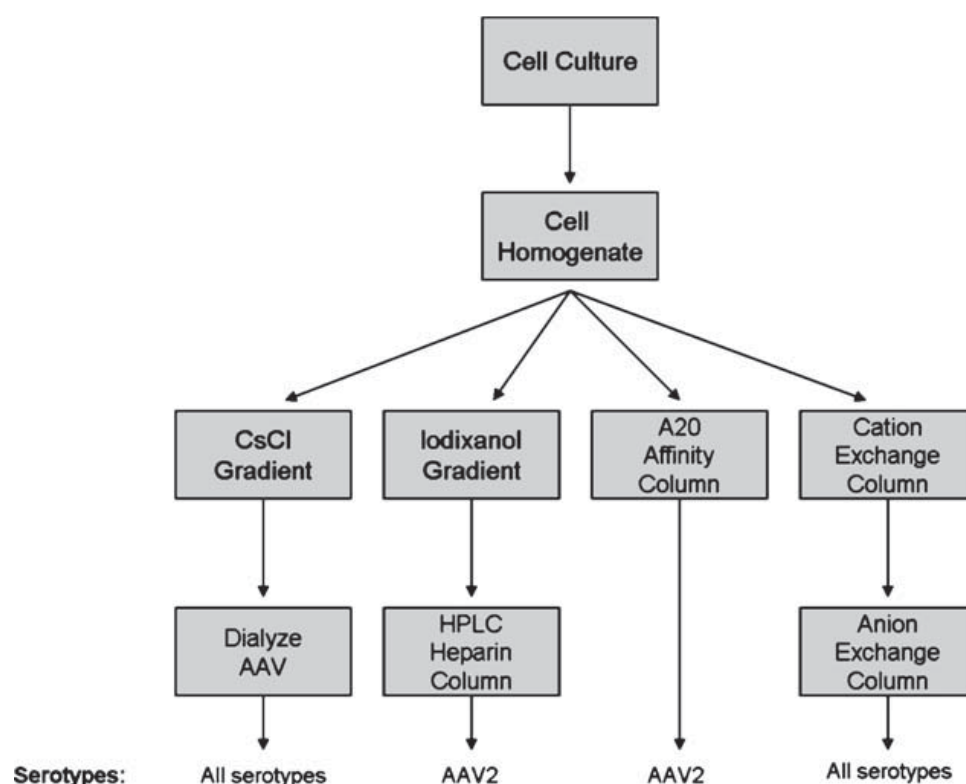


Fig. 3 A schematic representation of various methods (described in this chapter) used to purify rAAV from cell homogenate. The *bottom* of the schematic depicts the serotypes that can be purified using each method

methods are developed for generating high yields of rAAV, better methods of purification must be generated to acquire them in pure form. In addition, the biotechnology industry is building upon these numerous methods to produce rAAV by scaling up production utilizing large bioreactors containing liters of media and suspension cells, resulting in log increases of vector yield over the conventional small-scale methods described above.

3

Vector development

3.1

AAV2 Crystal structure

The structure of AAV2 has been determined at 3-Å resolution by X-ray crystallography [64], as shown in Fig. 4A. The AAV virion is composed of 60 capsid subunits arranged with $T = 1$ icosahedral symmetry. AAV contains three capsid proteins (Vp1, Vp2, and Vp3) in proportions of 1 : 1 : 8 that share overlapping sequences differing only at their *N* termini. Vp2 is 137 amino acids shorter than

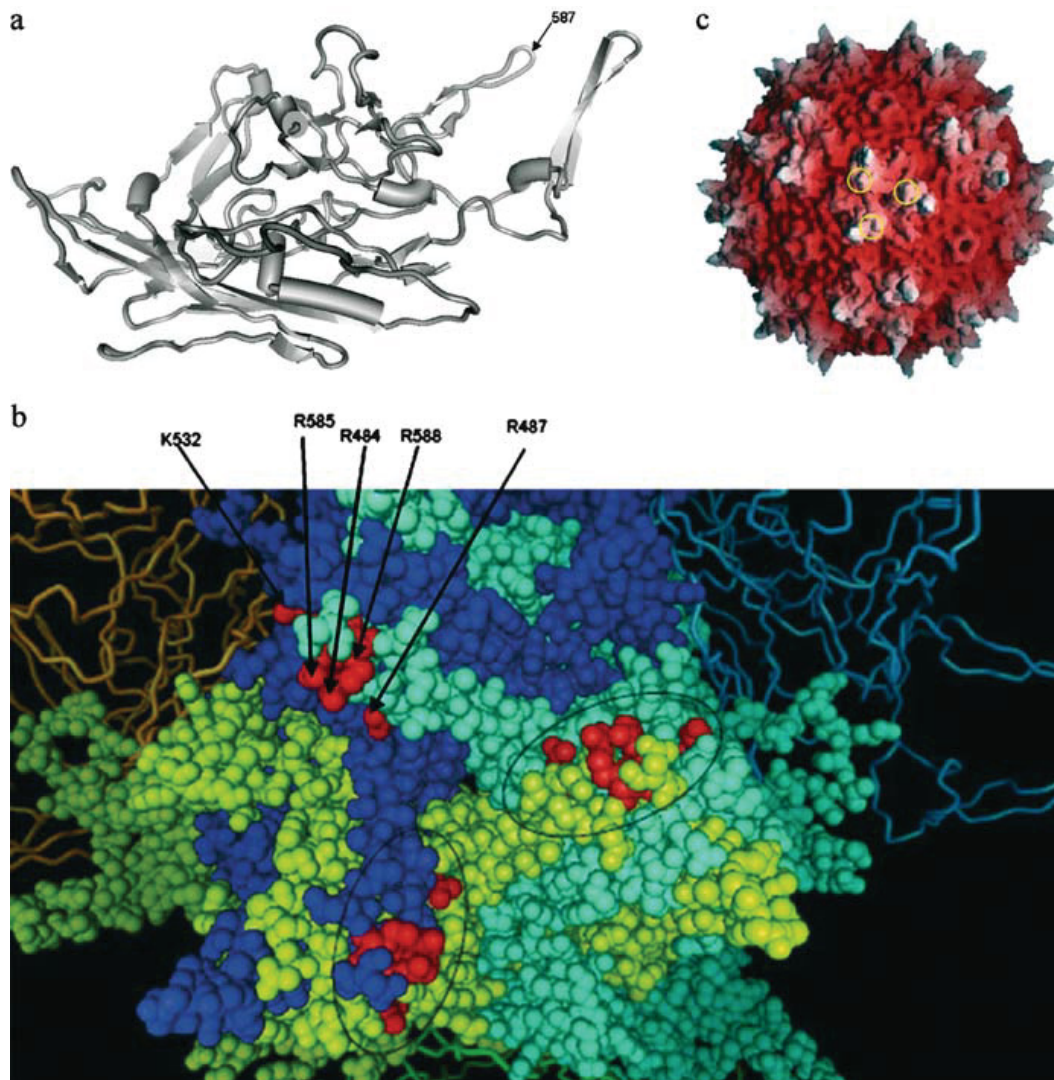


Fig. 4 Ribbon, space-filling, and cryo-EM diagrams of AAV2. **a** The crystal structure of the carboxy-terminal 519 amino acids of the AAV2 monomer subunit. N-terminal regions of Vp2 and Vp1 were not detected in the crystal structure. The amino acid (587) pointed to by an *arrow* represents the region of the monomer subunit where the majority of the successful retargeting ligands have been inserted. **b** A space-filling model of the three-fold axis of symmetry. Amino acids highlighted in *red* represent the amino acids necessary for heparan sulfate proteoglycan binding. R585 and R588 are supplied from one subunit and R484, R487, and K532 are supplied by a second subunit. Regions that are *circled* represent the other peaks that make up the three-fold spikes. **c** Depicts a cryo-EM image of the surface topology of AAV. The *yellow circles* identify the locations of the HSPG binding domain and the region where targeting ligands have been inserted. The authors would like to thank Dr. Michael Chapman for generously providing this image

Vp1 and is the product of an alternative start codon, while Vp3 is 65 residues shorter than Vp2. The 533 residue Vp3 is only visualized in the crystal structure, along with the corresponding regions of Vp1 and Vp2 occupying equal

positions in the capsid, but not the unique N-termini of Vp1 and Vp2. It is possible to produce Vp3-only viruses [7] that can successfully package and protect its genome and bind and internalize into cells. However, these viruses are not infectious (meaning they are incapable of trafficking to the nucleus) potentially due to the lack of the phospholipase domain [15] present in the unique N-terminus of Vp1 and/or an NLS sequence.

The basic subunit structure of AAV is comprised of a jelly-roll β -barrel motif that comprises two antiparallel β -sheets. Long loop insertions between the strands comprise about 60% of the structure. These interstrand loops contain β -ribbons and other additional secondary structures that constitute the surface features of AAV. These, in turn, govern interactions with antibodies and cellular receptors. The most prominent feature of the surface topology of AAV is the three-fold proximal peaks clustering around each three-fold axis of symmetry, as shown in Fig. 4C. Loops from neighboring subunits interact to form the peaks. Located at the center of each peak is a subloop that is part of the GH loop of one subunit that is inserted between two other subloops from the GH loop of an adjacent three-fold symmetry subunit producing the three-fold peaks [64].

The crystal structure of AAV2 will provide the knowledge needed to analyze the existing mutagenesis data and to direct future retargeting modifications of AAV2 and the other serotypes. In the past, genetic modification of AAV2 virions has depended on random mutagenesis [65], alanine-scanning mutagenesis [66], and site-directed insertional mutagenesis [66–68]. All of these strategies utilized the crystal structure of canine parvovirus [69] (CPV) along with the amino acid alignments of AAV2 with CPV as a guide for mutagenesis. These strategies have determined surface-displayed domains and regions that interfere with primary receptor binding (HSPG). The genetic data acquired from epitope mapping and mutagenesis studies has now been integrated with the crystal structure to form a rational picture that links function with structure.

Prior to the AAV2 crystal structure being solved, HSPG was determined to be the primary receptor for AAV2 [61]. The disaccharides that make up heparan and heparan sulfate can be modified by N sulfation as well as 2-O and 6-O sulfation to give an overall negative charge at physiological pH. Therefore, heparan sulfate can interact with numerous proteins, primarily by electrostatic attraction between the sulfate groups and clusters of positively charged amino acids [70, 71]. Alanine-scanning mutagenesis conducted by Wu et al. identified the amino acid domain of 585–588 as essential for HSPG binding [66]. Amino acids R585 and R588, depicted in Fig. 4B, located on one of the peaks at the three-fold axis of symmetry, were thought to be the only amino acids necessary for HSPG interaction until two recent studies [72, 73] elucidated three more basic amino acids essential for this interaction. It was possible to narrow down the amino acids to target mutagenesis utilizing the crystal structure of CPV and later that of AAV2 with

amino acid alignments of AAV serotypes 1–5. Both studies identified the basic amino acids of K532, R484, R487, R585, and R588 to be essential for HSPG interaction at the cell surface. K532, R487, R484 were found to play minor roles in HSPG binding while R585 and R588 were identified to be the most important. Figure 4B illustrates that all five amino acids are located in close proximity to one another, lining the canyon of the three-fold axis of symmetry.

3.2

Retargeting of rAAV2 to specific cell types

rAAV vectors possess a number of attractive features that include a relatively low pathogenicity, ability to transduce both dividing and nondividing cells, and long-term transgene expression in vitro and in vivo. rAAV vectors have been used to attain expression of a large number of genes in a variety of cells and organs including muscle, brain, eye, liver, and lung [1–4]. However, its broad host range is a disadvantage for in vivo gene therapy because it does not allow for tissue-specific transduction. Thus, efforts have been ongoing to target the well-characterized AAV2 based vectors to specific receptors.

At least two different strategies/methods are possible to achieve alternate receptor targeting of AAV [74]. The authors of this review [74] refer to these two different strategies as indirect and direct targeting. Indirect targeting is described as the interaction between the rAAV vector and the target cell, that is mediated by an associated molecule which is bound to the viral capsid and interacts with a cell surface receptor. Direct targeting involves the insertion of a ligand into the capsid structure that mediates cell-specific targeting of the vector.

When utilizing indirect targeting, knowledge of the three-dimensional structure of the virus is not necessary when high-affinity viral surface binding molecules (such as antibodies) are available. The bispecific molecules must bind to cell-specific receptors to allow uptake and proper intracellular viral processing. The success of this strategy is dependent upon the stability of the interaction of the virus with the bispecific molecule.

Targeting AAV2 using a bispecific antibody mediating the interaction between the virus and target cell was first described by Bartlett et al. (1999) [75]. The two monoclonal antibodies used to produce the bispecific molecule were AP-2 and A20. Heterodimers were generated by chemically cross-linking the Fab' arms, one with specificity toward $\alpha_{IIb}\beta_3$ integrin (AP-2 antibody) and the other with specificity toward the intact capsid (A20 antibody). Fibrinogen is the ligand for $\alpha_{IIb}\beta_3$ integrin and it becomes internalized via endocytosis. Thus, AAV2 was expected to become internalized via receptor-mediated endocytosis similarly to wtAAV2 infection when targeted to this integrin. The targeted vector was able to transduce MO7e (human megakaryocytic leukemia cell line) and DAMI (human megakaryoblastoid cell line) cells that

have been characterized as nonpermissive to wtAAV2 infection. The targeted AAV2 vector was able to transduce these cells 70-fold more efficiently. A subsequent study utilized a high-affinity biotin-avidin interaction as a molecular bridge to cross-link targeting ligands (EGF and FGF1 α) genetically fused to core-streptavidin [76]. This strategy was chosen because the avidin-biotin complex represents the highest affinity interaction between a ligand and a protein known in nature. Conjugation of the bispecific targeting protein to the capsid was accomplished by biotinylating purified rAAV2. The modified vectors, targeted to epidermal growth factor receptor (EGFR) or fibroblast growth factor 1 α receptor (FGFR1 α), resulted in an increase (> 100-fold) in transduction efficiency of EGFR positive SKOV3.ip1 (human ovarian cancer cell line) cells. The issue that remains to be resolved is the ability of these viruses to target similar cell types *in vivo* and to assess the stability of the virus-bispecific antibody complexes.

Four important constraints are required for the generation of targeting vectors via modifications of the capsid. The first is to identify the site of insertion on the capsid to place the targeting ligand to ensure that DNA packaging is unaffected and the ligand is exposed on the surface of the virus. Utilizing the crystal structure of AAV2, it is now possible to make educated guesses at identifying the regions of the capsid to insert targeted ligands. The second constraint involves modifying the vector so that it can no longer bind to its native cellular receptor(s), becoming dependent upon the targeting ligand for infection. The HSPG binding domain has been mapped to the GH loops located at the three-fold axis of symmetry on AAV2 [72, 73]. Therefore, it would be possible to insert a targeting ligand into this region to ablate the vectors' ability to bind HSPG and consequently retarget the vector to a specific cell type. The third constraint is the choice of the targeting peptide. It is complicated to determine the secondary structure of the ligand after insertion into the capsid. Thus, choosing a small structure-independent ligand is preferable to avoid destabilization of the capsid. The fourth constraint is that the ligand should target the vector to a receptor for internalization in a way that allows efficient transport of the virus and release of the viral DNA in the cell nucleus.

The first demonstration of a genetic modification leading to retargeting of AAV2, prior to the solving of the crystal structure, was described by Girod et al. [77]. They identified six sites on the capsid via a sequence alignment of AAV2 with CPV that were expected to be exposed on the surface of the capsid without disrupting functions essential to the viral life cycle. The ligand inserted into these six sites was the 14 amino acid L14 peptide that contains an RGD motif of the laminin fragment P1, which is the target for several integrin receptors. The L14 insertion into amino acid region 587 (depicted in Fig. 4A) was successful for retargeting AAV2 to infect mouse melanoma and rat schwannoma cell lines, which are incapable of binding to and infection by wtAAV2. In another study, amino acid region 587 was also used for insertion of an endothelial-specific peptide (SIGYPLP) that generated an AAV2

vector capable of infecting human umbilical vein endothelial cells and human saphenous vein endothelial cells [78]. Grifman et al. also utilized the insertion site at amino acid 587 to place the Myc epitope and the CD13 ligand (NGRAHA) [68]. Again, successful retargeting of AAV2 was achieved by gaining the ability to transduce Kaposi's sarcoma and rhabdomyosarcoma cell lines. These studies identified that insertion of a ligand into the amino acid 587 region inhibited AAV2 from binding its native receptor HSPG and confirmed that infection of the target cells is HSPG-independent. The peptide ligands that were chosen for these studies were identified by phage display prior to insertion into the capsid. Other noteworthy studies that were successful at retargeting AAV to cell lines of interest are detailed in references [79–84].

A study conducted by Wu et al. generated 93 mutants at 59 different positions on the AAV2 capsid to characterize functional domains on the AAV2 capsid [66]. Through their mutagenesis, regions were identified on the surface of the capsid that could possibly accept the insertion of a ligand. The regions identified were amino acids 266, 328, 447, 522, 553, 591, 664 in Vp3 as well as 34 in Vp1 and the *N*-terminus of Vp2 (138). The unique discovery in this study was that placing a serpin ligand at positions 34 and 138 of Vp1 and Vp2 respectively enhanced the ability of AAV2 to infect IB3 cells. This study and another study by Warrington et al. illustrated that insertions, as large as GFP, into the *N*-terminus of Vp2 are exposed on the surface of the capsid [16]. It is probable that targeting ligands placed in this region will enable AAV to target specific cell lines of interest. Mutations abrogating the HSPG binding domain at the three-fold axis of symmetry may be necessary to increase the specificity of the retargeting effort when positioning ligands at the *N*-terminus of Vp2.

Recently, a novel approach was developed that aided in the selection process of ligands to retarget AAV2 to specific cell types [85]. A random AAV2 peptide library was generated in which each particle displays a random 7-mer peptide at the capsid surface at amino acid position 588. This library was produced in a three-step process that ensured encoding of the displayed peptides by the packaged DNA. The diversity of the produced library of plasmids was 1.1×10^8 clones per library. Transfection of the library into producer cells would produce chimeric capsids displaying more than a single type of peptide insert and the packaged capsid gene would not encode the capsid mutants displayed on the viral surface. This would impede the selection process and the identification of selected capsid mutants. To resolve this, the library containing mutant capsid genes was packaged into capsids composed partially of wt capsid protein by cotransfecting wt rep-cap plasmid (pXX2) along with the AAV plasmid library. These particles were referred to as “AAV library transfer shuttles”. Shuttle vectors were purified by heparin columns at a level of about 60% of wild-type AAV2. 293T cells were then infected with an MOI of 1 and 5 per cell. Infecting with a low MOI with the shuttle vectors led to the production of randomized AAV capsid library particles that contained the corresponding cap gene. The selection process then begins by infecting a cell

line(s) of interest with the randomized AAV capsid library and Ad to amplify internalized AAV library clones. In this study, the cell line of interest was coronary endothelial cells. Amplified AAV from these cells were harvested and subjected to two more rounds of selection to enrich with AAV particles that successfully infect coronary endothelial cells. DNA was isolated from the cell lysate and AAV to PCR amplify the region containing the ligand to identify the sequence(s) necessary to infect the cell line of interest. A similar study was conducted to achieve AAV2 vectors targeted to cardiac tissue [86]. In vivo studies were carried out in the mouse via intravenous injection to assess luciferase activity in the heart. Transgene expression was five-fold higher in the hearts of animals injected with the targeted vector when compared to animals injected with the wt capsid rAAV2. This novel vector targeting strategy is advantageous over other approaches, such as phage display, in that selection happens within the context of viral capsid. Once the library has been generated, it is possible to screen numerous cell lines of interest to identify library clones that are efficient at targeting them.

It will be important to the field of gene therapy to develop targeting schemes to generate AAV2 vectors that could potentially be injected systemically, lacking its native tropism and having the ability to target the cell type(s) of interest. The aforementioned studies have established the groundwork for this concept. To further aid in targeting, tissue-specific promoters will need to be utilized to add another level of safety. It has also been shown that AAV-mediated transduction can be negatively impacted after internalization into the cell by proteasome degradation [87] and second strand synthesis [88]. Therefore, a better understanding of intracellular processing of AAV targeted vectors will be necessary, because these vectors may be transported into cellular compartments from which they will never escape or are processed in ways that prevent nuclear processing or gene expression.

3.3

Clades of AAV

Despite the longevity of transgene expression achieved by AAV2, its application has been limited by low levels of transgene expression in some tissues. This has been attributed to blocks at the level of cell binding and entry and intracellular processing. Progress in circumventing these issues has been made through the development of vectors based on other previously described serotypes that enter the cells through the use of receptors distinct from those utilized by AAV2. Intracellular processing and kinetics of uncoating may also be different for each serotype [89, 90]. AAV gene therapists have redirected their focus by integrating the other serotypes in preclinical studies using animal models to characterize the tropisms of each serotype. Each serotype has been shown to be advantageous over another based on their ability to transduce similar or different tissues more efficiently and/or evade the immune system.

Through work conducted by Gao et al., new serotypes of AAV have been identified (AAV 7, 8 and 9) along with a total of 108 new and unique isolates from 55 human and 53 nonhuman primates [91–93]. Table 1 illustrates capsid amino acid sequence diversity among AAV serotypes 1 through 9. The goal of their studies was to isolate new AAV serotypes with advantageous characteristics such as evasion of the human immune system (isolating AAV from nonhuman primates) and unique tropism. These new isolates were identified using PCR screening techniques on 259 human samples of 18 different tissue types derived from 250 individuals. Nonhuman primate data was gathered from similar tissues isolated from rhesus monkeys, cynomolgus and pigtailed macaques, baboons, and chimpanzees. Sequence variations in the isolated AAVs were identified as being located in hypervariable regions (HVRs) on the surface of the virus. Nine of the HVRs were mapped to the existing crystal structure of AAV2 in the Vp3 region. The three-fold proximal peaks, identified as the location of HSPG binding for AAV2, were found to contain the majority of the variability. Utilizing sequence alignments, they were able to generate a phylogenetic tree describing the evolution/homologous recombination phenomenon and to place the 108 isolates into nine clades. It is believed that the mechanism for the detected evolution seems to be a high rate of homologous recombination between different parental viruses infecting the same cell. The result is extensive swapping of HVRs of the capsid gene sequence, resulting in a variety of hybrids that could potentially have different tropisms and serology. This can be described as nature's system of "direct targeting" to generate tissue-specific AAV.

One of the main advantages to utilizing serotypes derived from nonhuman primates is their ability to evade the human immune system. This would allow the use of AAV serotype vectors in patients carrying neutralizing antibodies

Table 1 Capsid amino acid homology among AAV serotypes 1 through 9. AAV4 and AAV5 share the least homology with the other serotypes

Serotype	AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9
AAV1	100	83.3	86.8	63	57.9	99.2	84.8	83.9	82.2
AAV2	83.3	100	87.9	59.9	57.3	83.4	82.4	82.8	81.7
AAV3	86.8	87.9	100	62.8	58.4	87	84.8	85.7	83.6
AAV4	63	59.9	62.8	100	52.5	62.7	63.4	63.1	62.2
AAV5	57.9	57.3	58.4	52.5	100	58	57.7	57.6	56.7
AAV6	99.2	83.4	87	62.7	58	100	85	84	82.1
AAV7	84.8	82.4	84.8	63.4	57.7	85	100	87.8	81.8
AAV8	83.9	82.8	85.7	63.1	57.6	84	87.8	100	85.3
AAV9	82.2	81.7	83.6	62.2	56.7	82.1	81.8	85.3	100

to various AAV serotypes, either from acquired infection with the virus, or from previous treatment with serotype vectors. AAV serotypes may be distinct enough from each other to allow repeated patient treatment with two or more different members of the AAV family. The majority of the serotypes (AAV1, 2, 3, 5, 6, and 9) that have been characterized to date have been detected and isolated from human tissue. It has been shown in studies that neutralizing antibodies to some of these serotypes have been found in the human population. For example, Xiao et al. and Hildinger et al. screened 77 and 85 normal human subjects, respectively, for the presence of neutralizing antibodies (nAb) to AAV1 and 2. They concluded that approximately 20% had nAbs to AAV1 and 27% to AAV2 [94, 95]. nAbs have also been detected for AAV5 in the human population [96, 97]. Numerous studies carried out on rAAV vectors in animals and nonhuman primates have established that both humoral and cell-mediated immune responses can limit sustained transgene expression [4, 98, 99]. An interesting stipulation to these conclusions is that the cellular immune response to rAAV appears to be dependent upon the route of administration [100].

As described, it was recognized by researchers that efficient and controlled gene transfer to target cells and evasion of the host immune system will be crucial to the success and advancement of the gene therapy field. It is plausible that the development of targeted tissue-specific vectors from serotypes other than type 2 will be generated for therapies that depend on readministration of vector. This will allow for continued treatment of patients by evading neutralizing antibodies produced from previous treatments. It is apparent that the challenges for targeted gene transfer have been acknowledged and an established groundwork for addressing them has been recognized. With the ability to PCR from tissue unique AAV capsid sequences and the advent of the targeting field, an exciting future of AAV vector development for gene therapy is on the horizon.

4

Preclinical and clinical studies

4.1

Preclinical studies

The availability of high-titer, highly purified rAAV preparations has allowed for testing of rAAV gene delivery in many in vivo settings, including numerous rodent and nonhuman primate models. However, these studies support the need to develop alternative AAV serotypes as vectors since they possess advantageous characteristics over rAAV2 vectors. The next few paragraphs will give a brief overview of the preclinical data using rAAV serotypes as vectors followed by human clinical applications that have occurred or are ongoing.

Preclinical studies utilizing AAV serotypes 1 through 9 have lead to the identification of serotypes with advantageous characteristics over AAV2, such as tissue tropism and efficiency of transduction. The use of alternative AAV serotypes for transduction of the central nervous system [1, 101], skeletal muscle [92, 93, 95, 102, 103], liver [7, 95, 104, 105], eye [5, 7], pancreas [106–108], heart [109], and lung [110–113] have been met with success and are described in detail in their respective references.

The development of several disease models in mice and larger animals such as Parkinson's, Alzheimer's, hemophilia, diabetes, obesity, alpha-1-anti-trypsin deficiency, Canavan disease, cystic fibrosis, lysosomal storage diseases, Duchenne muscular dystrophy, and so on, have the potential to lead to new breakthroughs in rAAV vector application. Initial preclinical studies in some of the above disease models have progressed to the clinic for human therapy applications.

4.2

Clinical trials

To date, 25 Phase I and II human clinical trials have been set up using AAV as a vector. The majority of the human trials have been associated with cystic fibrosis (7) and the rest are as follows: hemophilia B, prostate and melanoma cancers, Canavan disease, amyotrophic lateral sclerosis (ALS), alpha-1-antitrypsin, Alzheimer's, Parkinson's, muscular dystrophy, rheumatoid arthritis, and HIV vaccines [121].

The first rAAV human gene therapy clinical trials began in 1995 under the direction of Dr. Flotte and Johns Hopkins University on cystic fibrosis [114]. The disease results from mutations in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl^- channel in the apical membrane of most secretory epithelia. This study utilized rAAV-CFTR vectors for administration to the respiratory tract and the vector was found to be well-tolerated in human patients. There was a trend to indicate that the vector was biologically active for the correction of the chloride channel [115, 116]. To date, there have been four phase I and three phase II clinical trials that have utilized rAAV-CFTR for treatment of CFTR in human patients. Phase II clinical trials found that the vector was well-tolerated with multiple doses utilizing different routes of administration (maxillary sinus and aerosol) [117, 118].

A phase I clinical trial was recently finished on hemophilia B [119]. Hemophilia B is an X-linked bleeding disorder (coagulopathy) caused by mutations leading to loss of function of coagulation factor IX. A dose-escalation study on adult men with severe hemophilia B (Factor IX < 1%) with rAAV ranging from 2×10^{11} vector genomes (vg)/kg to 1.8×10^{12} vg/kg injected at multiple intramuscular sites showed no evidence of toxicity up to 40 months after injection. Southern blot and immunohistochemical analysis of muscle

biopsies taken two and ten months after injection confirmed gene transfer into muscle. Circulating levels of Factor IX were detected to be less than 2% and most less than 1%. It was concluded from this study that more extensive transduction of muscle will be required to reach above 1% Factor IX circulating levels and the doses of rAAV tested were safe. Another phase I clinical trial of hemophilia B carried out by Avigen involved direct infusion of rAAV-Factor IX into the liver at three increasing dose levels. Subjects one through four received the low doses of rAAV and all of them tolerated the procedure well with no side effects. These subjects were unable to sustain Factor IX levels above 1%. One subject receiving the higher dose achieved circulating levels of Factor IX in excess of 10% of normal for about four weeks and in week four experienced an elevation in the levels of two liver enzymes leading to a rapid decline in Factor IX levels. This lead Avigen to suspend further enrollment until further data was gathered and confirmed by the FDA [122]. It is important to note that a liver response similar to this has never been observed in any of the preclinical animal studies.

Currently, phase I clinical trials are ongoing for alpha-1-antitrypsin deficiency (AAT) [118] and Canavan disease (Robert Wood Johnson Medical School in NJ and UNC-Chapel Hill Gene Therapy Center). The AAT clinical trial is utilizing rAAV2 to deliver the human AAT gene intramuscularly. AAT deficiency in humans can lead to lung diseases as serious as emphysema. Four cohorts of three subjects are being used with a dose escalation between them ranging from 2.1×10^{12} vg/patient to 7×10^{13} vg/patient. To date, rAAV2-AAT was found to be safe with all vector doses tested in the human patients. Testing of rAAV1-AAT in the phase I clinical trials is also expected to occur [124]. The Canavan clinical trial is being carried out on a group of ten patients using rAAV2 mediated delivery of human aspartoacylase gene (ASPA). Canavan disease is a childhood neurodegenerative disorder. This autosomal recessive disease is caused by mutations or deletions in the ASPA gene. An increase in the substrate molecule, N-acetyl-aspartate occurs with the lack of functional ASPA. This results in spongiform degeneration of the brain. In this ongoing study, 9×10^{11} rAAV2 encoding human ASPA gene are surgically delivered to six sites of the brain in each human patient. Preliminary immunological data suggests that there are no deleterious effects of AAV2 in the brain or other organs. Expansion into phase II and III trials will be essential for ongoing monitoring of patients and further assessment of safety issues.

The Neurologix company was approved by the FDA for phase I clinical trials for the treatment of Parkinson's disease in August of 2002. The first Phase I trial of this treatment began in August 2003. rAAV2-GAD (glutamic acid decarboxylase) was delivered stereotactically to the subthalamic nucleus of the brain where Parkinson's disease is extremely overactive [123]. The patients will receive low, medium and high doses of the gene treatment, and will undergo brain scans, neurologic and motor tests for a year.

Ceregene is planning to initiate additional gene therapy clinical trials during the coming year, including the FDA-approved Alzheimer's disease clinical trial beginning October 1, 2004 (rAAV2-NGF-nerve growth factor) and Parkinson's disease (rAAV2-GDNF-Glial cell line Derived Neurotrophic Factor), also expected to begin during 2004. A clinical trial in amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) is expected to be initiated in 2005 [125].

5

Conclusion

The intricate biology of AAV as a nonpathogenic human virus has led to its development into a promising viral vector. As a better understanding of AAV biology becomes available, we will see a direct impact on vector development (such as the determination of AAV receptors and viral tropism). The long-term potential of this vector will only be realized when the action of this reagent in humans is understood completely.

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